

AD _____

Award Number: DAMD17-99-1-9219

TITLE: An Estrogen Receptor-Selective Coactivator:
A Potential Regulator of Tamoxifen Effectiveness in Breast
Cancer Treatment and Prevention

PRINCIPAL INVESTIGATOR: Ramji R. Rajendran

CONTRACTING ORGANIZATION: University of Illinois
Champaign, Illinois 61820-6242

REPORT DATE: September 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation

20010815 125

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 2000	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Sep 99 - 31 Aug 00)	
4. TITLE AND SUBTITLE An Estrogen Receptor-Selective Coactivator: A Potential Regulator of Tamoxifen Effectiveness in Breast Cancer Treatment and Prevention			5. FUNDING NUMBERS DAMD17-99-1-9219	
6. AUTHOR(S) Ramji R. Rajendran				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Illinois Champaign, Illinois 61820-6242 E-MAIL: rajendra@students.uiuc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) There is a strong correlation between the presence of the estrogen receptor protein in breast tumors and their likelihood of response to endocrine therapy. We aim to better understand estrogen receptor physiology and its role in tamoxifen effectiveness. We have cloned a novel protein that interacts with the estrogen receptor in a ligand dependent manner. Having isolated the full-length clone, we find that this protein represses, rather than enhances, the activity of the estrogen receptor. This 97 kDa novel protein decreases the magnitude of estrogen receptor transcriptional activity by up to 90% in reporter gene assays of transfected breast cancer cell lines. This protein has intrinsic repression activity on a constitutively active SV40 promoter when recruited to it. We also show that the HDAC blocking drug Trichostatin A also reverses the repressive activity of this protein on the estrogen receptor. Thus, this protein may work by histone deacetylation. Further understanding of the mechanism of repression by this protein will help elucidate its role in estrogen receptor action in breast cancer cells as it may assist in repressing estrogen receptor stimulation of breast tumors and enhance the effectiveness of tamoxifen as an antiestrogen.				
14. SUBJECT TERMS Breast Cancer, Estrogen Receptor, Transcriptional Coregulator, Estrogen, Tamoxifen				15. NUMBER OF PAGES 55
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5-8
Key Research Accomplishments.....	9
Reportable Outcomes.....	10
Conclusions.....	11
References.....	12
Appendices.....	Abstracts and Manuscript Attached

Introduction:

Endocrine therapy is the main means of treating breast cancer either as an adjuvant or with metastatic disease. This treatment is most effective when a tumor is shown to express the estrogen receptor. To better understand the mechanism of action of the estrogen receptor, we previously performed a yeast two-hybrid screen of a MCF7 breast cancer cell cDNA library to determine the proteins that interact with the estrogen receptor in this cancer cell line. In this screen, we were able to identify a clone, which interacted with the estrogen receptor when it is bound to estrogen, and found that the encoded protein regulated the transcriptional activity of the estrogen receptor in breast cancer cells. Although our preliminary work suggested that this protein was a coactivator of the estrogen receptor, isolation and further characterization of the full length (97 kDa) protein acts as a repressor of the estrogen receptor. Thus, we have renamed this protein p97. This repression is relieved with the addition of the selective histone deacetylase inhibitor Trichostatin A. Therefore, we believe that p97 represses the receptor by recruiting histone deacetylase proteins. Furthermore, we have been able to demonstrate the interaction of p97 with the estrogen receptor in both an *in vitro* GST-pulldown assay and *in vivo* with a mammalian two-hybrid system. Further characterization of this protein will allow for a better understanding of the mechanism of action of the estrogen receptor in breast cancer cells.

Body:

Our specific aims are (1) to better characterize the interaction and activity of the p97 protein on estrogen receptor transcriptional activity; (2) to determine the mechanism of action of this protein; and (3) to examine the physiology of this protein in antiestrogen resistant and nonresistant breast cancer cells. According to our Statement of Work, we have focused on the first two of these goals during the first year. First, we found upon isolation of the full length clone and analysis of the full length protein that the p97 protein actually represses the activity of the estrogen receptor instead of enhancing the transactivation of the estrogen receptor. p97 represses the transcriptional activity of the estrogen receptor in many different promoter contexts. p97 is able to repress estrogen receptor activity on different types of promoters and estrogen response elements. p97 represses the activity of ER on several different estrogen responsive promoter constructs such as pS2, thymidine kinase, and complement C3. The transcriptional activity of the complement C3 promoter is repressed to a greater extent than the pS2 or TK promoters. This suggests that p97 may have differential repressive activity depending on the promoter context. p97 is also able to repress the TGF β 3 promoter at which ER is tethered, rather than being directly bound to the DNA site. Given this new data, we are confident that p97 is able to repress the activity of the estrogen receptor in different promoter contexts. p97 represses the magnitude of transcriptional activity of the estrogen receptor alpha and beta by 60%-90% of the maximal activity without added p97. We also find that p97 has some ability to repress the transcriptional activity of the

progesterone receptor, glucocorticoid receptor, and retinoic acid receptor. Experiments comparing the receptor selectivity are under way.

One of our major goals in Specific Aim 1 of our proposal was to characterize the interaction between p97 and ER. We have shown by both GST-pulldown assay and Mammalian two hybrid assay that the C-terminal third of p97 interacts with the DEF region of the estrogen receptor in a ligand dependent manner. Furthermore, no interaction of p97 was observed with the ABC region of the estrogen receptor. This agrees with our original yeast two-hybrid screen, because a clone only containing the C-terminal region of p97 must be recruited to the ligand binding/activation function 2 (DEF domains) of the estrogen receptor upon ligand binding to repress the transcriptional activity of the estrogen receptor. This interaction may thus serve as a means of regulating the estrogen receptor activity in breast cancer cells.

We have also focused our attention on determining the mechanism of action of the p97 protein over the past year as outlined in Specific Aim 2 of my research proposal. To determine if p97 may itself have the ability to directly repress ER, a chimeric protein was engineered that adds the Gal4 DNA binding domain to the amino terminus of p97. This chimera was then tested for its ability to repress a constitutively active SV40 promoter as a Gal4 chimera. We found that p97 has the ability to repress promoters by recruitment alone.

We have determined that the repression by p97 is independent of the N-terminal half of this protein. We will further dissect the repression domain of p97 by deletion analysis and determine exactly what region of this protein causes repression of transcriptional activity. p97 may be a direct acting corepressor that inhibits transcription

by being recruited to transcriptional activators and deacetylating histones to prevent access of the general transcriptional machinery. Further, we can determine if the amino acids that correspond to the repression domain of p97 also have intrinsic repression activity like the full-length protein.

In many cases, histone acetyltransferases are enzymes that enhance transcriptional activity, and histone deacetylases are enzymes that repress transcription. Trichostatin A is a drug that selectively inhibits histone deacetylase proteins. We have been able to show that Trichostatin A relieves the repressive action of p97 on ER transcriptional activity. Therefore, p97 either recruits histone deacetylase proteins or has intrinsic histone deacetylase activity. It is possible that p97 has histone deacetylase activity, but this is unlikely because p97 has no sequence homology to the highly conserved histone deacetylase family of proteins. Therefore, it probably recruits a complex of proteins including histone deacetylases.

The corepressors nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid receptor (SMRT) have been shown to interact with a complex containing mSin3a, HDAC1, and HDAC2 (Alland *et al.*, 1997, Heizel *et al.*, 1997). The mSin3a proteins were originally characterized by their interaction with the heterodimeric, basic helix-loop-helix repressor, Mad-Mxi1/Max. mSin3a has also been shown to work in concert with HDAC1 and HDAC2. The mSin3a/HDAC/SMRT complex can deacetylate histones and cause repression of nuclear receptors (Nagy *et al.*, 1997). It is likely that p97 recruits an HDAC complex like NCoR/SMRT do. We will determine if p97 interacts with known HDAC complexes by radioactively labeling the known proteins. We will then attempt to pull down these proteins with a GST fusion of the

repression domain-containing portion of p97. If Sin3a, HDAC1, or HDAC2 are pulled down by p97, then we can conclude that the protein may play a role in the transcriptional repression of p97.

Key Research Accomplishments for 9/1999 to 9/2000

- Obtained full-length clone of a novel protein originally isolated from a yeast-two hybrid screen
- Upon further characterization, the 97 kDa novel protein was found to act as a repressor of the estrogen receptor in many different estrogen-responsive promoter gene constructs such as pS2, thymidine kinase, and complement C3.
- Determined that the C-terminal region of p97 interacts with the DEF region (and not the ABC region) of the estrogen receptor in a ligand dependent manner
- Determined that p97 is able to work as an intrinsic repressor on constitutively active promoters
- Linked the ability of p97 to repress transcriptional activity of the estrogen receptor to histone deacetylase activity

Reportable Outcomes for 9/1999 to 9/2000

Manuscript:

Nye, A.C., Rajendran, R.R., Stenoien, D.L., Mancini, M.M., Katzenellenbogen, B.S., Belmont A.S. The estrogen receptor alters large-scale chromatin structure. Submitted to Mol. Biol. Cell, July 2000

Abstracts and Presentations:

Nye, A.C., Rajendran, R.R., Stenoien, D.L., Mancini, M.M., Katzenellenbogen, B.S., Belmont A.S. "The estrogen receptor alters large-scale chromatin structure." Poster presented at Dynamic Organization of Nuclear Function meeting. Cold Spring Harbor Laboratory, New York, Sept. 2000

Nye, A.C., Rajendran, R.R., Katzenellenbogen, B.S., Belmont A.S. "The estrogen receptor alters large-scale chromatin structure." Poster presented at Chromatin structure and DNA function meeting. Pennsylvania State University, July 1999.

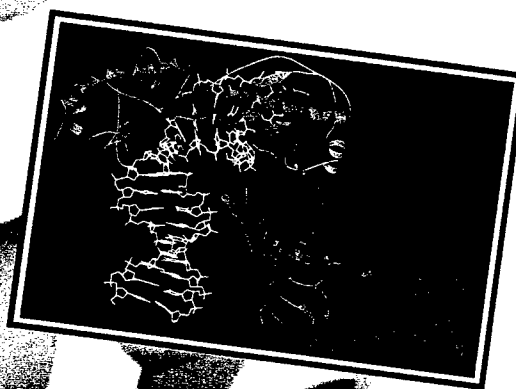
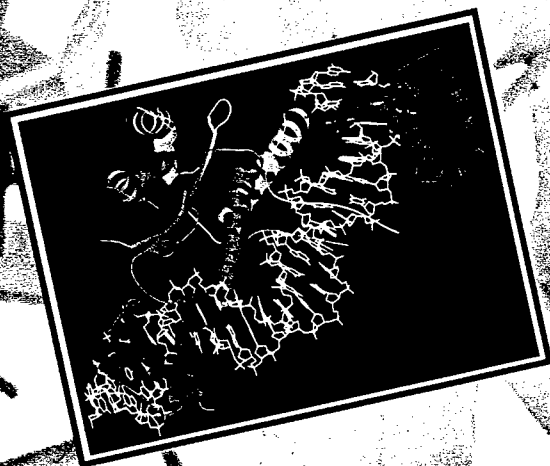
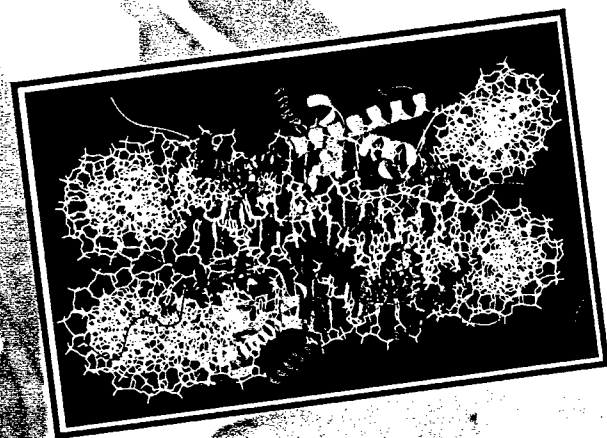
Conclusions:

During the first year of work we focused on Specific Aims 1 and 3. We have been able to determine that p97 is a novel protein that acts as a repressor of the transcriptional activity of the estrogen receptor. The C-terminal region of the p97 protein binds to the DEF region of the estrogen receptor. It may then be able to recruit histone deacetylase proteins to cause transcriptional repression of the estrogen receptor. Further studies are planned to verify this in breast cancer cells. Because p97 represses the activity of the estrogen receptor, it is plausible that changes in the level of p97 in breast cancers would affect the activity of their estrogen receptors. To determine this, we will now monitor the level of p97 in both antiestrogen sensitive and antiestrogen resistant cell lines. We will also use antisense-p97 approaches to monitor the consequence of p97 neutralization/ knocking out of the endogenous p97 in breast cancer cells.

References

- Alland, L., Muhle, R., Hou, H. Jr., Potes, J., Chin, L., Screiber-Augus, N., DePinho, R.A. (1997) Role for NCoR and histone deacetylase in mSin3-mediated transcriptional repression. *Nature* 387:49-55.
- Heinzel, T., Lavinsky, R.M., Mullen T.-M., Soderstrom, M., Laherty, C.D., Torachia, J., Yang, W.-M., Brard, G., Ngo, S.D., Davie, J.R., Seto, E., Eisenman, R.N., Rose, D.W., Glass, C.K., Rosenfeld, M.G. (1997) A complex containing N-CoR, mSin3a and histone deacetylase mediates transcriptional repression. *Nature* 387, 43-48.
- Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, Schreiber SL, Evans RM (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell*. 89:373-380.

Chromatin Structure and DNA Function: *Twenty-Five Years of the Nucleosome*



**Penn State's Eighteenth
Summer Symposium in
Molecular Biology**

PENNSTATE



**July 21-24, 1999
State College PA**

THE ESTROGEN RECEPTOR INDUCES DECONDENSATION OF LARGE SCALE CHROMATIN STRUCTURE; Anne C. Nye, Ramji R. Rajendran, Benita S. Katzenellenbogen, and Andrew S. Belmont; Department of Cell & Structural Biology, University of Illinois, Urbana-Champaign

The estrogen receptor was chosen as a model inducible transcriptional activator to study its effects on large-scale chromatin structure and nuclear organization. To accomplish this, the estrogen receptor was fused to the lac repressor DNA binding protein and tagged with GFP to allow visualization of amplified chromosome regions containing lac operator DNA sequences using a system developed by Robinett and others (1996). This fusion protein was capable of properly activating transcription in response to estradiol when tested on a transient CAT reporter plasmid containing lac operator sequences. When transfected into cells containing a large region of lac operator sequences integrated into the genome, the estrogen receptor fusion protein induced decondensation of the chromosomal region, causing the GFP-tagged chromosomal region to increase in area more than two-fold. The decondensed fibers formed by the estrogen receptor were thicker and less extended than those formed in previous experiments by the very strong VP16 transcriptional activator (T. Tumber, G. Sudlow, & A. Belmont, unpublished data). Surprisingly, decondensation of chromatin by the estrogen receptor was not dependent on the presence of the inducing hormone, estradiol. This preliminary data may be consistent with a two-step model for transcriptional activation which proposes "opening" of chromatin as the first step, followed by transcriptional activation itself. Studies with constructs containing truncated estrogen receptor and estrogen receptor mutants should help to clarify the role of this decondensation of large-scale chromatin structure.

Abstracts of papers presented
at the 2000 meeting on

DYNAMIC ORGANIZATION OF NUCLEAR FUNCTION

September 13–September 17, 2000

Arranged by

Thomas Cremer, *Ludwig-Maximilians University, Germany*
Robert Goldman, *Northwestern University*
Pamela Silver, *Dana Farber Cancer Institute*
David Spector, *Cold Spring Harbor Laboratory*



Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

THE ESTROGEN RECEPTOR ALTERS LARGE SCALE CHROMATIN STRUCTURE

Anne C. Nye*, David L. Stenoien#, Ramji R. Rajendran*, Benita S. Katzenellenbogen*, Michael A. Mancini#, and Andrew S. Belmont*

*University of Illinois at Urbana-Champaign

#Baylor College of Medicine

Whether higher order chromatin structure influences transcriptional regulation remains unclear. Recently we demonstrated an unfolding of large-scale chromatin fibers after targeting a GFP-lac repressor-VP16 transcriptional activation domain to a heterochromatic chromosome arm containing the lac operator repeats. Here we apply a similar approach to target the estrogen receptor (ER) to heterochromatin and euchromatin. GFP-lac repressor was fused to wild-type ER, the ABC domains of ER containing the weak constitutive activation function 1 (AF-1), and the DEF domains of ER containing wild-type or mutant forms of the ligand-inducible activation function 2 (AF-2). The ABC domains of ER were found to alter chromatin structure in a small proportion of cells, producing novel thin, protruding fibers unlike any previously observed. The full length ER fusion and the DEF fusion both induced slight unfolding of a heterochromatic chromosomal region in the presence of the inducing hormone, estradiol. Surprisingly, decondensation of chromatin was much more dramatic in the absence of estradiol, tripling the average size of the unfolded region and approaching within twofold the average size induced by VP16. Similar fibers resulted when ER was targeted to both heterochromatic and euchromatic sites, and these fibers appeared to involve unfolding of lower levels of chromatin structure than those observed with lac repressor-VP16. Remarkably, these significantly unfolded fibers of chromatin re-condensed within 30 minutes of adding estradiol, as viewed in living cells. These results have implications for mechanisms of steroid receptor action.

THE ESTROGEN RECEPTOR ALTERS LARGE SCALE CHROMATIN STRUCTURE

Running title: Estrogen receptor & chromatin structure

ANNE C. NYE*, RAMJI R. RAJENDRAN*, DAVID L. STENOIEN+, MICHAEL A.
MANCINI+, BENITA S. KATZENELLENBOGEN*, AND ANDREW S. BELMONT*

* Department of Cell and Structural Biology, University of Illinois, Urbana-Champaign,
IL

+ Department of Cell Biology, Baylor College of Medicine, Houston, TX

Keywords: downregulation, transcription, recruitment assay, SRC-1

Address correspondence to: Andrew S. Belmont, B107 CLSL, 601 S Goodwin Ave,
Urbana IL, 61801. Phone: (217) 244-2311 fax: (217) 244-1648, email: asbel@uiuc.edu

Abstract

Whether higher levels of chromatin folding influence transcriptional regulation remains unclear. To observe the effects of the estrogen receptor (ER) on large-scale chromatin structure in mammalian cells, we targeted ER to chromosome arms containing lac operator repeats. GFP-lac repressor fusions to wild type ER and several truncations and mutations functioned as expected in transient transcription assays and demonstrated correct intranuclear localization and recruitment of the coactivator SRC-1. GFP-lac repressor-ER produced moderate changes in large-scale chromatin structure in response to estradiol, but in the absence of hormone the fusion produced dramatic unfolding. The size of the unfolded chromosome arm approached that seen with the 100-fold stronger transcription activator VP16, demonstrating that the strength of the activator is disproportionate to the degree of unfolding. GFP-lac repressor-ER also produced less distinct fibers than VP16, suggesting that different activators may affect different levels of large-scale chromatin structure. The unfolding of chromatin by unliganded GFP-lac repressor-ER was rapidly reversed within 30 minutes of adding hormone. These changes are consistent with a model in which unliganded ER produces unfolded, poised chromatin. According to this model, addition of hormone leads to rapid initiation of transcription followed by downregulation and reversion of the chromatin to its original state.

Abbreviations used: AF, activation function; CAT, chloramphenicol acetyltransferase; CFP, cyan fluorescent protein; CHO, Chinese Hamster Ovary; DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; E₂, 17 β estradiol; ER, estrogen receptor; ERE, estrogen response element; GFP, green fluorescent protein; HSR, homogenously staining region; ICI, antiestrogen ICI182,780; lac rep, lac repressor; NLS, nuclear localization signal; SRC-1, steroid receptor coactivator 1; TOT, trans-hydroxytamoxifen; VP16 AAD, viral protein 16 acidic activation domain; YFP, yellow fluorescent protein;

Introduction

In recent years, a clear connection between transcriptional regulation and chromatin modification has emerged. Several biochemical mediators of this connection, including histone acetyltransferases, histone deacetylases, and chromatin remodeling complexes have been identified through genetic and biochemical means (Howe, et al., 1999, Peterson and Workman, 2000, Tyler and Kadonaga, 1999). In several model systems, gene activation is accompanied by local histone modifications, recruitment of remodeling complexes, and changes in nucleosome positioning. Such experiments have led to an increasing appreciation for the regulatory role imposed by local chromatin structure. What remains unknown is the degree to which higher levels of chromatin structure also impact transcriptional regulation, and whether changes in these higher levels of chromatin structure are mediated by protein complexes which also modulate local chromatin structure.

We have defined large-scale chromatin organization as corresponding to the folding of 10 and 30 nm chromatin fibers into interphase and mitotic chromosomes (Belmont, 1997, Belmont, et al., 1989). In previous work, we explored the capability of transcriptional activators to alter chromatin structure at this level of organization by targeting a lac repressor - VP16 acidic activation domain (VP16 AAD) fusion protein to heterochromatic chromosome regions containing large numbers of lac operator repeats. The targeting of this very strong transcriptional activator, derived from the herpes simplex virus, caused a dramatic unfolding of heterochromatic chromosome regions into extended, large-scale chromatin fibers (Tumbar, et al., 1999). This unfolding occurred even after inhibition of RNA pol II transcription and therefore is not the result of

transcription per se but results from chromatin modifications induced by trans factors recruited directly and/or indirectly by the VP16 AAD. Unexpectedly, the observed decondensation induced by the VP16 AAD largely involved the uncoiling or extension of ~100 nm diameter large-scale fibers, which we have termed chromonema fibers, without significant decondensation of these chromonema fibers into dispersed 30 or 10 nm chromatin fibers.

Given these dramatic effects on large-scale chromatin structure by VP16 AAD, we were interested in whether other types of transcriptional activators, particularly weaker, endogenous activators, might induce comparable conformational changes. Moreover, we were interested in whether the observed effects by other transcriptional activators would be proportional to the strength of the transcriptional activator. Finally, we were interested in whether different transcriptional activators would alter chromatin structure in qualitatively different ways.

In this paper, we analyze changes in large-scale chromatin structure induced by estrogen receptor (ER). ER is a member of the nuclear receptor superfamily, the largest class of eukaryotic transcription factors (Tsai and O'Malley, 1994), which activate transcription in response to ligands. Estrogen receptor is classified with other steroid hormone receptors as a Type I receptor. Steroid receptors share a modular structure consisting of six domains, denoted A through F, and they contain two activation functions: the constitutive AF-1 in the N-terminal A/B domains and the ligand-inducible AF-2 in the C-terminal E domain, with the DNA binding domain in the central domain C (Katzenellenbogen, et al., 1997).

Like all nuclear hormone receptors, the estrogen receptor works with a number of coregulators to affect transcription (reviewed in (Glass and Rosenfeld, 2000, McKenna, et al., 1999)). When overexpressed, coactivators can increase ER's transcriptional response to estradiol, and when some coactivators are knocked out, the response to hormone is greatly impaired (Xu, et al., 1998). Several classes of coactivators are proposed to work at the level of chromatin structure; most notably chromatin remodeling complexes and histone acetyltransferases. These include the coactivator hBrm, the human homolog of yeast chromatin remodeling complex factor SWI2/SNF2. Coactivators in the p160 family have histone acetyltransferase activity, including hSRC-1 (steroid receptor coactivator-1) and ACTR/p/CIP/AIB1/Rac3/TRAM1/SRC-3 (Chen, et al., 1997, Onate, et al., 1995, Spencer, et al., 1997). The more general coactivators p300, CBP, TAFII250, and P/CAF also exhibit histone acetyltransferase activity (Bannister and Kouzarides, 1996, Mizzen, et al., 1996, Ogryzko, et al., 1996, Yang, et al., 1996).

These coregulators indicate an important role for local chromatin structure in ER transcriptional activation. Here we describe our findings that the ER is in fact also capable of dramatically altering large-scale chromatin structure.

Materials and Methods

Construction of plasmids

To facilitate construction of estrogen receptor fusion proteins, a mammalian expression plasmid containing the GFP-lac rep-SV-40 nuclear localization signal (NLS) fusion under control of the F9-1 promoter (Tumbar, et al., 1999) was modified by Quik-change site-directed mutagenesis (Stratagene, La Jolla, CA), resulting in the insertion of a 9 bp fragment containing an AscI site into the PvuII site 7 amino acids N-terminal to the linker and NLS. This generated GFP-lac rep-AscI-NLS (NYE4). Selected regions of the estrogen receptor were amplified by PCR using primers which contain AscI sites in frame. The template for these reactions was either wild-type human estrogen receptor alpha from the CMV - ER plasmid (Wrenn and Katzenellenbogen, 1993), or mutant estrogen receptors (L525A or L540Q) in the same vector backbone (Ince, et al., 1993), (Ekena, et al., 1997, Schodin, et al., 1995). The PCR products were digested with AscI and ligated into the AscI-digested GFP-lac rep-AscI-NLS vector to create in-frame fusions. All regions of constructs which had undergone PCR were sequenced to ensure fidelity. DNA Strider v. 1.2 (Christian Marck, France), Gene Construction Kit (Textco, Inc.), and NCBI's BLAST website (<http://www.ncbi.nlm.nih.gov/BLAST>) were used to facilitate sequence analysis. Construction of a functional yellow fluorescent protein (EYFP, Clontech) fusion with steroid receptor coactivator-1 (YFP-SRC-1) has been described (Stenoien, et al., 2000). The CFP-lac repressor-ER fusion is described elsewhere (Stenoien, D.L., Nye, A.C., Mancini, M., Patel, K., Dutertre, M., Smith, C.L., Belmont, A.S., and Mancini, M.A., submitted).

The 8op-CAT reporter plasmid (NYE10) was generated by removing a BamHI - HindIII fragment containing 8 lac operator repeats from pPS-8.1 (Robinett, et al., 1996). This fragment was ligated into the pATC2 vector (Mattick, et al., 1997) whose two EREs had been removed with a BglIII/HindIII digestion.

Tissue Culture, Transfection, and CAT Assays

A03_1 CHO DG44 cells with stably integrated lac operator repeats (Li, et al., 1998) were cultured at 37° C with 5% CO₂ in F-12 HAM's media without hypoxanthine or thymidine, with 0.3 uM methotrexate, without phenol red, and with 10% dialyzed fetal bovine serum (HyClone Labs, Logan, UT) treated with charcoal/dextran. Phenol-red free trypsin was used to passage cells. RRE_B1 cells (S. Dietzel and A.S. Belmont, submitted) were cultured using the same medium except with 10 µM methotrexate. Wild type Chinese hamster ovary CHO-K1 cells (ATCC CRL#9618) were cultured in phenol-red free F-12 Ham's medium with 10% charcoal/dextran treated fetal bovine serum.

Transfections on coverslips were performed with FuGENE6 reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions using 250 ng DNA and 3 µL reagent per 35 mm plate. Fresh media containing hormone was added 24 hours after transfection. Seventy-two hours after transfection, cells were rinsed in calcium, magnesium free phosphate buffered saline (CMF-PBS), fixed in CMF-PBS with 1.6% paraformaldehyde (Polysciences, Warrington, PA), and stained with 0.2 µg/ml DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) in CMF-PBS. Transfections for CAT transcription assays used 1.6 µg 8op-CAT or pATC4 (4 ERE-TATA-CAT) reporter (Mattick, et al., 1997), 0.6 µg beta-galactosidase internal reference reporter pCH110

(Pharmacia), and 10 ng of effector plasmid combined with 15 uL of FuGENE6 reagent per 60 mm plate. Fresh media containing hormone was added 24 hours post-transfection and cells were harvested and lysed 48 hours post-transfection. CAT assays were performed as described (Schodin, et al., 1995), and normalized for beta-galactosidase expression.

Fluorescence Microscopy & Image Analysis

Images were collected on an inverted light microscope (IMT-2, Olympus, Success, NY) equipped with a cooled, slow-scan CCD camera (Photometrics, Tucson, AZ) as described previously (Hiraoka, et al., 1991). Optical sections of nuclei were collected and deconvolved as described (Agard, et al., 1989). Figures were assembled using Adobe Photoshop. ScionImage software (Scion Corp., Frederick, Maryland) was used to measure the area of the lac operator containing homogeneously staining regions (HSRs) within nuclei. A macro based on the "Analyze particles" command was used to measure the cross-sectional area of each HSR defined using a single, grey level threshold. Proper thresholding was verified by visual inspection and defined interactively, if necessary, using the freehand selection tool. Measurements were exported, analyzed, and graphed in Microsoft Excel and SigmaPlot.

Results

Experimental Design

In these experiments, we used a system developed recently to allow visualization of changes in large-scale chromatin structure which accompany targeting of proteins to specific chromosome regions (Tumbar, et al., 1999). Lac repressor protein was fused with estrogen receptor (ER) and green fluorescent protein (Fig. 1A), and this fusion protein was expressed in the Chinese Hamster Ovary (CHO) A03_1 cell line. A03_1 cells contain an amplified chromosome arm (Fig. 1B) containing pSV2-DHFR-8.32 vector repeats; each vector copy contains 256 direct repeats of the lac operator and the DHFR (dihydrofolate reductase) transgene, spaced by large blocks of co-amplified genomic DNA. The average vector repeat consists of ~30 copies and the average block of co-amplified DNA is ~1000 kb (Li, et al., 1998). The amplified chromosome region, also called a homogeneously staining region (HSR), is ~90 Mbp in total size, and has properties of heterochromatin. The HSR appears as a condensed mass roughly 1 μ m in diameter through most of interphase, and it replicates in middle to late S phase. Targeting a control GFP-lac repressor fusion protein produces no obvious conformational changes in the amplified chromosome region; however, targeting a GFP-lac repressor protein fused with the acidic activation domain of the strong transcriptional activator, VP16, has been shown to produce a dramatic unfolding of this heterochromatic structure (Tumbar, et al., 1999).

To study the effects of the estrogen receptor (ER) on large-scale chromatin structure, we made several GFP-lac repressor fusion proteins containing different regions of the estrogen receptor (Fig. 1C). GFP-lac rep-ER contains full-length human estrogen

receptor alpha. The GFP-lac rep-ABC construct contains the three N-terminal domains of ER that encompass the weak constitutive transcriptional activation function AF-1 in the A/B domains and ER's DNA binding domain in the C domain. The GFP-lac rep-DEF of ER fusion contains the three C-terminal domains, including the ligand binding domain and the estradiol-inducible transcriptional activation domain AF-2. Two mutations were introduced into the DEF fusion. The L525A mutation virtually eliminates estradiol binding, greatly diminishing transcription activation (Ekena, et al., 1996) (Ekena, et al., 1997). The L540Q mutation, which is in a critical coactivator binding portion of AF-2, still binds estradiol with normal affinity but shows markedly reduced transcriptional activation because of the impaired recruitment of coactivators (Ince, et al., 1993, Schodin, et al., 1995, Wrenn and Katzenellenbogen, 1993).

Nuclear redistribution, transcriptional activation, and coactivator recruitment by ER fusion proteins after hormone addition

The GFP-lac rep-ER fusion protein retains normal ER activity based on three different criteria.

First, expression of the GFP-lac rep-ER fusion protein in wild type CHO cells shows a homogeneous nuclear distribution in the absence of hormone which shifts to a nonhomogeneous reticular distribution after estradiol addition (data not shown). This behavior is indistinguishable from that previously reported for a GFP-ER fusion (Htun, et al., 1999, Stenoien, et al., 2000). In comparison, both the GFP-lac repressor and GFP-lac rep-VP16 AAD fusion proteins show homogeneous nuclear distributions which do not change with estradiol addition (data not shown).

The intracellular localization of ER truncations has not previously been studied. The GFP-lac rep-ABC fusion protein (a deletion of the ligand-binding domain) showed a homogeneous nuclear distribution independent of estradiol concentration. The GFP-lac rep-DEF fusion protein showed a homogeneous nuclear distribution in the absence of hormone, which changed to a speckled pattern with estradiol. These speckles were unlike the reticular, sometimes called "hyperspeckled" pattern produced by full-length ER because the speckles were typically larger and more distinct, though speckle size varied between different transfected nuclei. These speckles did not colocalize with staining for a splicing factor, SC-35, which localizes to speckles of similar morphology (data not shown). Point mutants in the GFP-lac rep-DEF construct corresponding to ER L525A, which does not bind ligand well, and ER L540Q, which shows impaired coactivator recruitment, showed homogeneous nuclear staining independent of estradiol concentration. These results imply that hormone dependent redistribution of ER depends on functional ligand binding and a functional AF-2 region of ER. For all fusion proteins, with and without estradiol, there are occasional cells with fusion protein localizing to a fibrillar aggregation as previously observed for GFP-ER in other cell types (Htun, et al., 1999).

Second, using a transient expression reporter system, GFP-lac rep-ER and GFP-lac rep-DEF fusion proteins showed hormone dependent transcriptional activation with a dose response similar to that of wild type ER. For these assays, we constructed a reporter plasmid containing 8 lac operator binding sites upstream of a TATA box followed by the CAT reporter gene (Figure 1C). The 4ERE-TATA-CAT reporter (pATC4) is identical except that it contains 4 consensus ERE's instead of 8 lac operators. Dose response

curves for transcriptional activation by GFP-lac rep-ER and GFP-lac rep-DEF binding to lac operators are similar to the dose response of wild type ER binding to ERE's, with a response peaking or reaching a plateau at 10^{-8} or 10^{-9} M estradiol (Fig. 2A) Therefore, all experimental treatments with estradiol used the hormone at 10^{-9} M.

The level of transcriptional activation at 10^{-9} M estradiol from the GFP-lac rep-ER fusion protein, expressed from the F9-1 promoter and using the 8 lac op reporter construct, was only 2.5 fold lower than wild type ER, expressed from the stronger CMV promoter using the 4 copy ERE reporter construct (direct comparison not shown). Comparisons of levels of transcription at 10^{-9} M estradiol for the different fusion proteins are shown in Fig. 2B. GFP-lac rep-ABC, which lacks the receptor hormone binding domain E, showed a very weak level of transcription which was not increased by hormone, and transcription from the two mutants of ER (L525A and L540Q) and from the negative control GFP-lac rep were less than 10% that of GFP-lac rep-ER. GFP-lac rep-VP16 AAD transcriptional activation was roughly 150 times higher than GFP-lac rep-ER, using the same promoter to drive fusion protein expression (data not shown).

Third, a cyan fluorescent protein-lac rep-ER fusion protein (CFP-lac rep-ER), bound to lac operator repeats, shows in vivo, hormone-enhanced recruitment of a YFP-SRC-1 coactivator fusion protein. The construction and functional testing of these fusion proteins is described elsewhere (Stenoien, et al., 2000 and Stenoien, D.L., Nye, A.C., Mancini, M., Patel, K., Dutertre, M., Smith, C.L., Belmont, A.S., and Mancini, M.A., submitted). Consistent with recent FRET results in vivo (Llopis, et al., 2000), there is a slight recruitment of YFP-SRC-1 at the CFP-lac rep-ER bound HSR in the absence of hormone, with a significant amount of YFP-SRC-1 distributed throughout the nucleus

(Fig. 3). Quantitative measurements reveal that within minutes of adding estradiol, absolute levels of YFP-SRC-1 at the HSR increase significantly, while nuclear background levels of YFP-SRC-1 show an absolute decrease.

Changes in large-scale chromatin structure after targeting of ER and truncated ER fusion proteins

GFP-lac rep-ER fusions were transiently transfected into A03_1 cells (Li, et al., 1998). Transfection of GFP-lac rep-ER and addition of estradiol causes some cells' GFP-labeled chromosome regions to decondense slightly compared to the control, but most HSR's qualitatively resemble the control (Figure 4A, top left). In these experiments, estradiol was added at a timepoint 24 hrs after transfection and 48 hrs before cell fixation. Quantitative measurements on large numbers of transfected cells reveals a slight shift towards larger HSR sizes in the histogram comparison of GFP-lac rep-ER transfected cells relative to GFP-lac repressor transfected control cells (Fig. 5a), with the mean HSR area increasing from $1.06 \mu\text{m}^2$ to $1.41 \mu\text{m}^2$.

Unexpectedly, in the *absence* of estrogen a significant fraction of the cell population transfected with GFP-lac rep-ER contains chromosomal regions which decondense significantly into expanded structures (Fig. 4A) whose sizes are frequently similar to those seen with the GFP-lac rep-VP16 AAD fusion protein with or without estradiol (Fig. 4B). Quantitatively, the mean area increases to roughly three times the control area with GFP-lac rep-ER in the absence of hormone ($3.07 \mu\text{m}^2$), compared to over five times the control area with the GFP-lac rep-VP16 AAD fusion protein ($5.46 \mu\text{m}^2$, Fig. 5). HSR's larger than $2.7 \mu\text{m}^2$ were considered "open", because this threshold

counts nearly all of the negative control HSR's as condensed and correlates well with qualitative observations. By this criteria, 39% of HSRs with GFP-lac rep-ER were open, compared to ~70% of HSRs using GFP-lac rep-VP16 AAD and 3% of HSRs with the control GFP-lac repressor constructs. More detailed statistics for all of the constructs tested is provided in Fig. 5B.

Although the deconvolved images of the HSR structures produced by GFP-lac rep-ER in the absence of hormone are suggestive of some underlying fiber-like structures, the fibers appear significantly less distinct than typically seen after decondensation by GFP-lac rep-VP16 AAD. This suggests a qualitative difference in the decondensed structures produced by the VP16 and ER fusion proteins. To ensure that these differences were not due to fixation conditions, we performed microscopy on living cells. Two examples for each construct are shown side by side, with the deconvolved images shown on top and the original, raw data shown below (Fig. 4C). With the GFP-lac rep-VP16 AAD construct the fibrillar nature of the HSR is apparent even in the raw, unprocessed image. However, in the raw images of the GFP-lac rep-ER construct, the HSR appears much more homogeneous, with a texture suggestive of fibrillar staining only in the deconvolved images.

Qualitative and quantitative results were very similar using the GFP-lac rep-DEF construct (Fig. 4A & 5B). The ER mutants L525A and L540Q lack estradiol-dependent activity as anticipated and closely resemble the structures seen with wild type GFP-lac rep-DEF and GFP-lac rep-ER in the absence of estradiol.

The GFP-lac rep-ABC construct, containing the weak, constitutive AF-1 transcriptional activation domain, produced a small, ~50% increase in average HSR area.

This increase was mainly due to a subpopulation of cells, around 10% both with or without estradiol treatment, in which GFP-lac rep-ABC caused a dramatic unfolding (Fig. 4A&C). This unfolding was qualitatively different from anything seen with either the GFP-lac rep-VP16 AAD, GFP-lac rep-ER, or GFP-lac rep-DEF constructs. Specifically, these cells show thin, extended fibers protruding several microns out from a more densely folded core. These were seen in both fixed (Fig. 4A) and live cells (Fig. 4C).

Increase in heterochromatic HSR size induced by ER fusion proteins is rapidly reversed in vivo by estradiol addition

In the experiments described above, samples containing estradiol were exposed to hormone 24 hrs after transfection and 48 hrs before fixation. HSRs remained relatively condensed with the GFP-lac rep-ER or GFP-lac rep-DEF fusion proteins, differing slightly from the control HSRs exposed to GFP-lac repressor. Under these conditions, estradiol treatment could lead to condensed HSR's in two ways: by blocking the decondensation of chromatin induced by the ER fusion proteins in the absence of hormone, or by causing re-condensation of structures that had already opened. To distinguish between these possibilities, A03_1 cells were transfected with GFP-lac rep-ER and fixed 72 hrs later. Estradiol was added at varying time points preceding fixation. Statistical comparison of HSR areas indicated a rapid decrease in HSR size occurring in less than one hr after estradiol addition (data not shown).

To follow this re-condensation process more carefully, we carried out direct observations on living cells transfected with GFP-lac rep-ER before and after addition of estradiol. 3-D optical section data stacks were collected from cells at timepoints

following addition of estradiol. Individual sections from the deconvolved data stacks are shown for three examples in Fig. 6A. Data sets with more frequent timepoints can be viewed as Quicktime movies at the online version of this journal (Fig. 6B). A striking recondensation of large-scale chromatin structure occurred within minutes of estradiol addition in 10 out of 15 cells (66%) observed in this manner. Based on comparison with the statistics collected from analysis of fixed cell preparations, exposure to UV light in the process of collecting these 4-D images appeared to inhibit this condensation somewhat. We therefore limited UV exposure by taking individual optical sections at each timepoint and found condensation to occur in 10 out of 10 cells (100%).

Condensation was complete within 30 minutes and the HSRs showed minimal further change in structure through the end of the experiment, up to 3 hours later. Condensation occurred with minimal changes in nuclear position or shape; the HSRs did not appear to fold over long distances as they condensed, but rather decreased in size uniformly, perhaps due to local condensation of individual large-scale chromatin fibers. Major structural features such as the arm-like projections in the bottom example (Fig. 6A) were retained during the condensation.

To better estimate the change in HSR area over time, we carried out similar experiments but took only single optical sections at each time point, to minimize UV exposure. The average change in area 30 minutes after estradiol addition was ~50% (Table I). The antiestrogens, trans-hydroxytamoxifen (TOT) and ICI182,780 (ICI), produced comparable changes in HSR area.

Estradiol treatment induces appearance of more distinct large-scale chromatin fibers

In addition to the global condensation caused by hormone, we also noticed a local condensation of large-scale chromatin structure and the appearance of distinct, large-scale chromatin fibers within several minutes of adding estradiol to cells expressing GFP-lac rep-ER (Fig. 6A). At light microscopy resolution, the diffuse fibrillar appearance of these HSRs became more well-defined, becoming very similar to the distinct fibers normally observed with the GFP-lac rep-VP16 AAD fusions.

A non-heterochromatic, decondensed HSR shows no significant changes in HSR area and only local changes in large-scale chromatin structure after estradiol treatment

In A03_1 cells the HSR is normally condensed and late replicating. By these criteria, the HSR behaves as heterochromatin. The extensive condensation of the HSR observed with GFP-lac rep-ER after hormone treatment could therefore be explained in two ways. Addition of hormone might induce an active, chromatin condensing activity of the ER itself. Alternatively, addition of hormone might simply reverse or downregulate whatever large-scale chromatin decondensation activity is produced by ER targeting, with the global condensation of the HSR to a smaller area simply representing a reversion to the HSR's normal, heterochromatic state. We also considered the possibility that dimerization of ER in response to ligand might cause GFP-lac rep-ER fusions to aggregate and therefore appear to condense chromatin.

To distinguish between these possibilities, we examined changes in HSR morphology occurring in cells expressing GFP-lac rep-ER after hormone treatment using a different cell line, RRE_B1. The HSR conformation in this cell line is variable, but has a very high percentage of cells with unusually extended and fibrillar HSRs, relative to the bulk large-scale chromatin structure seen throughout most of the nucleus (Belmont and Bruce, 1994). It is likely that much of the variability in HSR conformation between different cells is cell cycle related. These open, fibrillar HSRs are observed using lac repressor staining in fixed cells which do not express endogenous lac repressor (not shown) and in vivo in cells expressing GFP-lac repressor (Fig. 7, right). Therefore, this RRE_B1 cell line displays the most euchromatin-like HSR of all lines produced in our laboratory.

In RRE_B1 cells expressing GFP-lac rep-ER, addition of estradiol does not lead to significant changes in HSR area, either for the more highly extended or the more compact HSRs within this cell line (Fig. 7). Thus, it is unlikely that the condensation seen in the other cell line, A03_1, is due to dimerization or aggregation of GFP-lac rep-ER fusion proteins in response to ligand. We therefore conclude that the global condensation of HSRs in the presence of hormone in the other cell line, A03_1, represents a reversal or down-regulation of ER activity, allowing the HSR to revert to its normal heterochromatin state.

We note, however, that GFP-lac rep-ER does produce some change in the RRE_B1 cells' HSRs in response to estradiol. As shown in Fig. 7, RRE_B1 cells expressing GFP-lac rep-ER show very open HSR's with a fluffy fibrillar appearance. Because of the high variability of the structures in control cells expressing GFP-lac

repressor, we cannot determine easily whether this structure differs from that seen with GFP-lac repressor alone. Yet, upon addition of estradiol to GFP-lac rep-ER expressing RRE_B1 cells, we see a local condensation in which the fuzzy fibers change to more sharply demarcated, distinct fibers (see arrows, Fig. 7). These local changes in HSR structure are quite similar to those described above for the A03_1 cells (Fig. 6).

Discussion

We have described changes in large-scale chromatin organization induced by targeting a functional estrogen receptor fusion protein to amplified chromosome regions. Binding of GFP-lac rep-ER and GFP-lac rep-DEF fusion proteins produces a 20-40% increase in area of a heterochromatic amplified chromosome region after long term estradiol exposure. Surprisingly, much larger increases in area were produced by the full length ER and the AF-2-containing DEF fusion proteins in the *absence* of hormone. Despite the fact that ER's transcriptional potential is much weaker than the activator VP16, the global expansion of chromatin fibers in many GFP-lac rep-ER transfected cells in the absence of ligand were comparable to that produced by VP16.

These open HSRs condensed significantly within 30 minutes of hormone addition. In contrast, adding hormone to targeted ER fusions at a normally open, "euchromatic" amplified chromosome arm from the RRE_B1 cell line did not lead to significant decreases in area. These results suggest that upon hormone treatment, ER-bound chromatin condenses towards the level normally seen for that chromatin in the absence of ER and estrogen, ruling out that ER has a powerful chromatin-condensing activity or that condensation is due to dimerization or aggregation of the receptor. Further evidence that dimerization does not cause aggregation is that the GFP-lac rep-DEF L540Q mutant should be capable of binding hormone and dimerizing (Ince, et al., 1993, Schodin, et al., 1995), yet it does not allow the chromatin to condense.

We have also shown that different transcriptional activators can produce qualitatively different unfolded chromatin fibers. In previous work, VP16 AAD produced

an increase in HSR area through an extension or uncoiling of large-scale chromatin fibers; these fibers straightened but did not change noticeably in condensation level (Tumbar, et al., 1999). In contrast, in the absence of hormone, the GFP-lac rep-ER and GFP-lac rep-DEF fusion proteins produced an increase in HSR area that was accompanied by the loss of distinct, well demarcated fibers at light microscopy resolution. These diffuse fibers were also observed when ER fusion proteins were targeted to the RRE-B1 "euchromatic" HSR. The GFP-lac rep-ABC fusion protein containing the AF-1 activation domain produced a decondensation pattern qualitatively different from any of the other ER or VP16 AAD fusion proteins. Extended fibers could be seen protruding out from a more densely packed core, as if each fiber was pulled out from the core, or attached at its end to some nuclear substructure.

Biological Significance

It is reasonable to propose that at least some transcriptional activators known to induce remodeling of lower levels of chromatin organization also induce remodeling of higher levels of chromatin structure. The system we have developed now allows us to probe for such changes in higher levels of chromatin structure. Although this system with its multitude of targeting binding sites is obviously far from the situation found in endogenous promoters, the amplification it provides allows sensitive detection of perturbations in large-scale chromatin organization. Our working hypothesis is that similar structural perturbations may be produced over smaller neighborhoods surrounding endogenous promoters.

Assuming this is the case, our results indicate that in the absence of hormone the estrogen receptor recruits factors leading to a decondensation of large-scale chromatin structure. These factors can antagonize the condensation produced in heterochromatic chromosome regions, producing a global expansion of chromatin. In what may represent an independent activity, our results suggest that the estrogen receptor also leads to a local decondensation by at least partly unfolding these large-scale chromatin fibers, an effect not seen with the much stronger transcriptional activator VP16. Intriguingly, the local and global unfolding activities appear maximal in the absence of hormone, and do not require domains A-C of ER. In addition, the decondensation is not impaired by point mutations in the ligand binding domain (L525A) or in AF-2 (L540Q), nor by a truncation that eliminates helix 12 (CFP-lac-ER 534) (Stenoien, D.L., Nye, A.C., Mancini, M., Patel, K., Dutertre, M., Smith, C.L., Belmont, A.S., and Mancini, M.A., submitted). This suggests that a region between amino acids 262-534 is responsible for ligand-independent decondensation and that decondensation is not sufficient for transcriptional activation. The qualitatively distinct HSR morphology produced by targeting the AF-1 region of ER suggests a possibility for another type of decondensation.

Hormone treatment leads to a local recondensation of large-scale fibers within 5-30 minutes. For HSRs that are normally condensed, there is also significant global recondensation. We have interpreted the recondensation of large-scale chromatin structure observed after addition of hormone with ER targeting as a downregulation of decondensation activities. This downregulation could be due to proteasomal degradation (Lonard, et al. 2000) or inactivating modifications of ER coactivators, as shown recently for the acetylase ACTR (Chen, et al. 1999). Interestingly, the kinetics of downregulation

of decondensation activity are quite similar to the kinetics of downregulation of transcriptional activation observed for endogenous ER-responsive genes. These kinetics have been determined by nuclear run-on assay for several genes. Pro-cathepsin-D peaks around 2.5 fold induction between 30 minutes and 2 hours after adding estradiol to cells in culture, and is downregulated to 1.5 fold at 2 hours (Cavaillès, et al., 1988). Initiation of c-myc transcripts peaks between 5 minutes and 30 minutes after adding estradiol to cells in culture, and is downregulated to 2.5 fold by 2 hours (Dubik and Shiu, 1988). When estradiol is injected into rats rather than added to cells in culture, induction is somewhat slower: for c-jun, jun-D, and c-fos, and jun-B, initiation of transcripts peaks between 90-120 minutes and is downregulated to near-basal levels by 4 hours (Cicatiello, et al., 1992). Similar results are seen for glucocorticoid receptor activation from the MMTV promoter, indicating that this downregulation may be common for steroid hormone receptors (Archer, et al., 1994, Ucker and Yamamoto, 1984). Of particular note is a recent study of several endogenous estradiol responsive genes which showed accumulated mRNA levels, RNA Pol II engagements of the promoter, acetylation of histones H3 and H4, and coactivator binding all increase within 30 minutes to an hour and are downregulated thereafter (Chen, et al., 1999). Moreover, changes in intranuclear estrogen receptor dynamics and coactivator association also occur on a timescale of minutes after hormone treatment (Stenoien, et al., 2000).

In our system, ER was artificially tethered to DNA sites independent of hormone presence. The simplified, prevailing view to date appears to be that ER is homogeneously localized within the nucleus in the absence of hormone and binds to its target ERE elements only after hormone addition. In this case our observations of large-scale

chromatin decondensation by unliganded ER in our tethered system may represent a snapshot of the early stages of liganded ER binding EREs. If, however, unliganded ER *does* bind to EREs, our observations are even more physiologically relevant.

In fact, increasing evidence indicates that at least some ER does bind to EREs in vivo in the absence of hormone. This derives from promoter interference assays in intact mammalian cells (Reese and Katzenellenbogen, 1992), as well as chromatin immunoprecipitation assays with mammalian cell extracts (Chen, et al., 1999). Both studies demonstrated significant ER binding in the absence of hormone, although estradiol was shown to enhance ER binding to EREs several fold. In the latter study, ER remained bound to EREs even when the promoter shut down as measured by decrease in mRNA production, decrease in PolII initiation, and loss of histone acetylation (Chen, et al., 1999).

Therefore, since there is evidence that significant levels of unliganded ER bind to EREs, our observations of large-scale chromatin decondensation suggest a two step model in which opening of large-scale chromatin structure by unliganded ER creates a poised state. Addition of hormone leads to rapid recruitment of additional coactivators and recruitment of RNA pol II holoenzyme, yielding a burst of transcription from this unfolded chromatin. This is followed by downregulation of large-scale chromatin decondensation and downregulation of transcriptional activation. Because antagonists can also recondense chromatin, we propose that they cause the downregulation of ER activity without the initial burst of transcription. Future extensions of the technology described here should allow us to test this two step model directly.

This work was supported by grants from the National Institutes of Health to A.S. Belmont (R01-GM58460 and R01-GM42516), M.A. Mancini (R01-DK55622) and B.S. Katzenellenbogen (CA 18119 and CA 60514); a National American Heart Association Scientist Development Award and funds from the Department of Cell Biology, Baylor College of Medicine to M.A. Mancini; a NIH postdoctoral fellowship (1F32DK09787) to D. Stenoien; a NIH Bioacoustics and Bioengineering in Radiation Oncology Traineeship (NIH T32 CA09067) and Department of Defense Predoctoral Traineeship (DAMD17-99-1-9219) to R. Rajendran; and a NIH Cell and Molecular Biology Traineeship (T32-GM07283) and a Howard Hughes Medical Institute Predoctoral fellowship to A. Nye.

References

- Agard, D. A., Hiraoka, Y., Shaw, P. and Sedat, J. W. (1989). Fluorescence microscopy in three dimensions. *Methods Cell Biol.* 30, 353-377.
- Archer, T. K., Lee, H. L., Cordingley, M. G., Mymryk, J. S., Fragoso, G., Berard, D. S. and Hager, G. L. (1994). Differential steroid hormone induction of transcription from the mouse mammary tumor virus promoter. *Mol. Endocrinol.* 8, 568-76.
- Bannister, A. J. and Kouzarides, T. (1996). The CBP co-activator is a histone acetyltransferase. *Nature* 384, 641-3.
- Belmont, A. S. (1997) in *Genome Structure and Function*, ed. Nicolini, C. (Kluwer Academic Publishers, Dordrecht), 261-276.
- Belmont, A. S., Braunfeld, M. B., Sedat, J. W. and Agard, D. A. (1989). Large-scale chromatin structural domains within mitotic and interphase chromosomes in vivo and in vitro. *Chromosoma* 98, 129-143.
- Belmont, A. S. and Bruce, K. (1994). Visualization of G1 chromosomes: a folded, twisted, supercoiled chromonema model of interphase chromatid structure. *J. Cell Biol.* 127, 287-302.
- Cavaillès, V., Augereau, P., Garcia, M. and Rochefort, H. (1988). Estrogens and growth factors induce the mRNA of the 52K-pro-cathepsin-D secreted by breast cancer cells. *Nucleic Acids Res.* 16, 1903-19.
- Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y. and Evans, R. M. (1997). Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* 90, 569-80.
- Chen, H., Lin, R. J., Xie, W., Wilpitz, D. and Evans, R. M. (1999). Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell* 98, 675-86.
- Cicatiello, L., Ambrosino, C., Coletta, B., Scalona, M., Sica, V., Bresciani, F. and Weisz, A. (1992). Transcriptional activation of jun and actin genes by estrogen during mitogenic stimulation of rat uterine cells. *J. Steroid Biochem. Mol. Biol.* 41, 523-528.
- Dubik, D. and Shiu, R. P. (1988). Transcriptional regulation of c-myc oncogene expression by estrogen in hormone-responsive human breast cancer cells. *J. Biol. Chem.* 263, 12705-8.

- Ekena, K., Weis, K., Katzenellenbogen, J. A. and Katzenellenbogen, B. S. (1996). Identification of amino acids in the hormone binding domain of the human estrogen receptor important in estrogen binding. *J. Biol. Chem.* 271, 20053-20059.
- Ekena, K., Weis, K. E., Katzenellenbogen, J. A. and Katzenellenbogen, B. S. (1997). Different residues of the human estrogen receptor are involved in the recognition of structurally diverse estrogens and antiestrogens. *J. Biol. Chem.* 272, 5069-75.
- Glass, C. K. and Rosenfeld, M. G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev.* 14, 121-141.
- Hiraoka, Y., Swedlow, J. R., Paddy, M. R., Agard, D. A. and Sedat, J. W. (1991). Three-dimensional multiple-wavelength fluorescence microscopy for the structural analysis of biological phenomena. *Sem. Cell Biol.* 2, 153-165.
- Howe, L., Brown, C. E., Lechner, T. and Workman, J. L. (1999). Histone acetyltransferase complexes and their link to transcription. *Crit. Rev. Eukaryot. Gene Expr.* 9, 231-43.
- Htun, H., Holth, L. T., Walker, D., Davie, J. R. and Hager, G. L. (1999). Direct visualization of the human estrogen receptor alpha reveals a role for ligand in the nuclear distribution of the receptor. *Molec. Biol. Cell* 10, 471-86.
- Ince, B. A., Zhuang, Y., Wrenn, C. K., Shapiro, D. J. and Katzenellenbogen, B. S. (1993). Powerful dominant negative mutants of the human estrogen receptor. *J. Biol. Chem.* 268, 14026-14032.
- Katzenellenbogen, B. S., Montano, M. M., Ekena, K., Herman, M. E. and McInerney, E. M. (1997). Antiestrogens: mechanisms of action and resistance in breast cancer. *Breast Cancer Res. Treat.* 44, 23-38.
- Li, G., Sudlow, G. and Belmont, A. S. (1998). Interphase cell cycle dynamics of a late replicating, heterochromatic homogeneously staining region: precise choreography of condensation / decondensation and intranuclear positioning. *J. Cell Biol.* 140, 975-989.
- Lonard, D. M., Nawaz, Z., Smith, C. L., O'Malley, B. W. (2000). The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Mol. Cell.* 5, 939-48.
- Mattick, S., Glenn, K., de Haan, G. and Shapiro, D. J. (1997). Analysis of ligand dependence and hormone response element synergy in transcription by estrogen receptor. *J. Steroid Biochem. Mol. Biol.* 60, 285-94.
- McKenna, N. J., Lanz, R. B. and O'Malley, B. W. (1999). Nuclear receptor coregulators: cellular and molecular biology. *Endocr. Rev.* 20, 321-44.

- McNally, J. G., Muller, W. G., Walker, D., Wolford, R. and Hager, G. L. (2000). The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. *Science* 287, 1262-5.
- Mizzen, C. A., *et al.* (1996). The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. *Cell* 87, 1261-70.
- Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H. and Nakatani, Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* 87, 953-9.
- Onate, S. A., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* 270, 1354-7.
- Peterson, C. L. and Workman, J. L. (2000). Promoter targeting and chromatin remodeling by the SWI/SNF complex. *Curr. Opin. Genet. Dev.* 10, 187-92.
- Reese, J. C. and Katzenellenbogen, B. S. (1992). Examination of the DNA-binding ability of estrogen receptor in whole cells: implications for hormone-independent transactivation and the actions of antiestrogens. *Mol. Cell. Biol.* 12, 4531-8.
- Robinett, C. C., Straight, A., Li, G., Willhelm, C., Sudlow, G., Murray, A. and Belmont, A. S. (1996). In vivo localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition. *J. Cell Biol.* 135, 1685-1700.
- Schodin, D. J., Zhuang, Y., Shapiro, D. J. and Katzenellenbogen, B. S. (1995). Analysis of mechanisms that determine dominant negative estrogen receptor effectiveness. *J. Biol. Chem.* 270, 31163-71.
- Spencer, T. E., *et al.* (1997). Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* 389, 194-8.
- Stenoien, D. L., Mancini, M. G., Patel, K., Allegretto, E. A., Smith, C. L. and Mancini, M. A. (2000). Subnuclear trafficking of estrogen receptor-alpha and steroid receptor coactivator-1. *Mol. Endocrinol.* 14, 518-34.
- Tsai, M. J. and O'Malley, B. W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* 63, 451-86.
- Tumbar, T., Sudlow, G. and Belmont, A. S. (1999). Large-scale chromatin unfolding and remodeling induced by VP16 acidic activation domain. *J. Cell Biol.* 145, 1341-54.
- Tyler, J. K. and Kadonaga, J. T. (1999). The "dark side" of chromatin remodeling: repressive effects on transcription. *Cell* 99, 443-6.

Ucker, D. and Yamamoto, K. (1984). Early events in the stimulation of mammary tumor virus RNA synthesis by glucocorticoids. *J. Biol. Chem.* 259, 7416-7420.

Wrenn, C. K. and Katzenellenbogen, B. S. (1993). Structure-function analysis of the hormone binding domain of the human estrogen receptor by region-specific mutagenesis and phenotypic screening in yeast. *J. Biol. Chem.* 268, 24089-24098.

Xu, J., Qiu, Y., DeMayo, F. J., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W. (1998). Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science* 279, 1922-5.

Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H. and Nakatani, Y. (1996). A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* 382, 319-24.

Ylikomi, T., Wurtz, J. M., Syvala, H., Passinen, S., Pekki, A., Haverinen, M., Blauer, M., Tuohimaa, P. and Gronemeyer, H. (1998). Reappraisal of the role of heat shock proteins as regulators of steroid receptor activity. *Crit. Rev. Biochem. Mol. Biol.* 33, 437-66.

I. Live recondensation induced by antiestrogens

% original size after 30 min.			
treatment	treatment	s.d.	n
10^{-9} M E_2	52%	22%	16
2×10^{-6} M TOT	40%	17%	23
2×10^{-6} M ICI	39%	12%	31
None	116%	27%	13

A03_1 cells which contain a heterochromatin-like HSR were transfected with GFP-lac rep-ER and cultured in the absence of estradiol. The cells were observed in a live cell chamber on the fluorescence microscope. Images were collected before adding 17β -estradiol at 10^{-9} M, trans-hydroxytamoxifen (TOT) at 2×10^{-6} M, or ICI182,780 (ICI) at 2×10^{-6} M and at each timepoint afterwards. The sizes of HSRs were measured as in Figure 4, and the size after 30 minutes was divided by the original size to determine the % original size.

FIGURE 1 – Experimental design

(A) Schematic of the GFP-lac repressor-estrogen receptor fusion protein used in this study, which binds to lac operator DNA sequences. (B) Schematic of the cell line used in this study, which has been previously described. A vector containing a 256-copy repeat of the lac operator DNA sequence and the dihydrofolate reductase gene (*dhfr*) was transfected into *dhfr*⁻ CHO cells. The vector was amplified in vivo by increasing concentration of methotrexate, an inhibitor of DHFR. This results in a large insert of lac operator vector repeats which are visible when GFP-lac repressor constructs are expressed. (C) Constructs made for this study.

FIGURE 2 – Transcriptional activity of ER fusion proteins

(A) Dose response assays of transcriptional activity of ER fusion proteins in CHO cells in response to varying concentrations of 17 β estradiol (E_2). The data are normalized to a β -galactosidase internal control reporter plasmid, and results are displayed relative to the response of each construct at 10^{-9} M E_2 . (B) Transcriptional activity of constructs, tested with or without 10^{-9} M 17 β estradiol (E_2). Results are displayed relative to the sample with the highest average activity, GFP-lac rep-DEF plus E_2 . The error bars depict the standard error of the mean, and in some cases are too small to be visible.

FIGURE 3 - CFP-lac rep-ER recruits YFP-SRC-1

A03_1 cells were cotransfected with CFP-lac rep-ER (top row) and YFP-SRC-1 (bottom row) and subjected to live microscopy during hormone addition. Shown are two nuclei side by side, each with one lac operator-containing array. Exposure conditions and image manipulations were the same for each panel to reflect the increase in brightness of YFP-SRC-1 following the recruitment. All images are deconvolved and represent a 3-D reconstruction of a series of z-sections. Bar=10 μ M.

FIGURE 4 - The estrogen receptor alters the appearance of large-scale chromatin fibers

(A and B) Deconvolved optical sections of fixed cells. DAPI staining is shown as blue and GFP as red in the left panel of each pair. A black and white close-up of each GFP labeled chromosomal region is shown in the right panel of each pair. The left side of each

row indicates the presence (+E₂) or absence (-E₂) of 10⁻⁹ M 17 β estradiol. (C) Optical sections of live cells. Top panel shows a deconvolved section and the bottom panel shows the raw image. Two examples are shown for each construct. Scale bars = 1 μ m.

FIGURE 5 - The estrogen receptor alters the size of HSRs

Data collection of areas of HSRs is described in the text. (A) Histograms show the percent of cells (y-axis) having each area of HSR (x-axis). (B) Boxplots display HSR size information for all populations studied. Ends of the lines show the 10th and 90th percentiles, ends of the boxes show the 25th and 75th percentiles, and the center line shows the 50th percentile (median). For the percent “open” column, HSRs were counted as “open” if they measured larger than 2.7 μ m².

FIGURE 6 – Live recondensation induced by estradiol in A03_1 cells

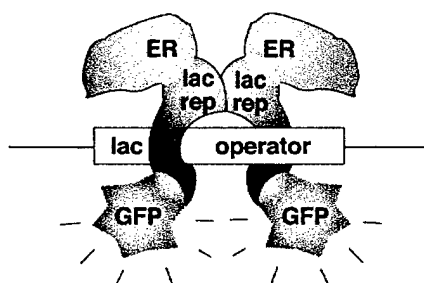
(A) A03_1 cells which contain a heterochromatic HSR were transfected with GFP-lac rep-ER and cultured in the absence of estradiol. The cells were observed in a live cell chamber on the fluorescence microscope. Optical sections were taken before adding 17 β -estradiol at 10⁻⁹M and at each timepoint afterwards. A deconvolved section is shown. (B) Still images showing the starting point for videos which are available at the online version of this journal. The images for each frame of the videos were obtained as described for (A) but with more frequent timepoints. Top cell: initial frame is taken prior to adding estradiol; each frame of the movie is 1 minute later for a total of 11 minutes. Bottom cell: initial frame is taken prior to adding estradiol; each frame of the movie is 2 minutes later for a total of 30 minutes. Scale bar = 1 μ m.

FIGURE 7 - Live recondensation induced by estradiol in RRE_B1 cells

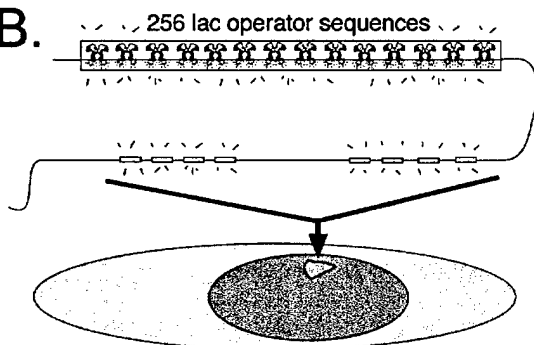
RRE_B1 cells which contain a euchromatin-like HSR were transfected with GFP-lac rep-ER and treated as described in Figure 6. For comparison, an example of the negative control GFP-lac repressor is shown to the right, though this cell line shows great variability. Arrows emphasize examples of fibers which condense locally. Scale bars = 1 μ m.

Figure 1

A.



B.



C. Constructs made

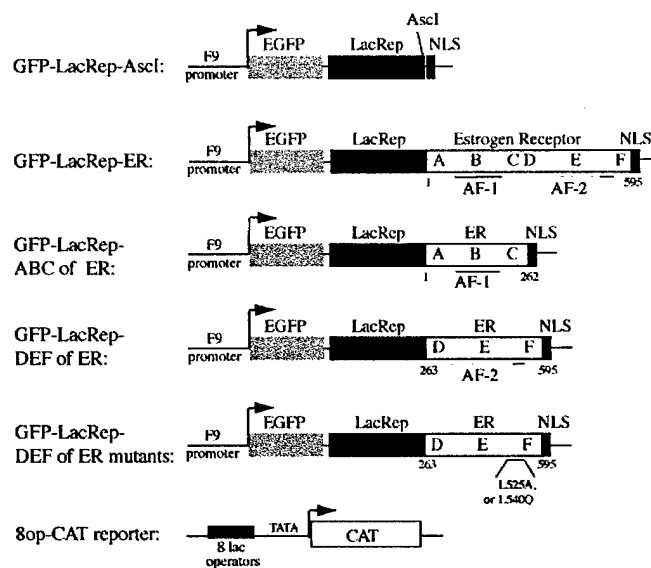


Figure 2

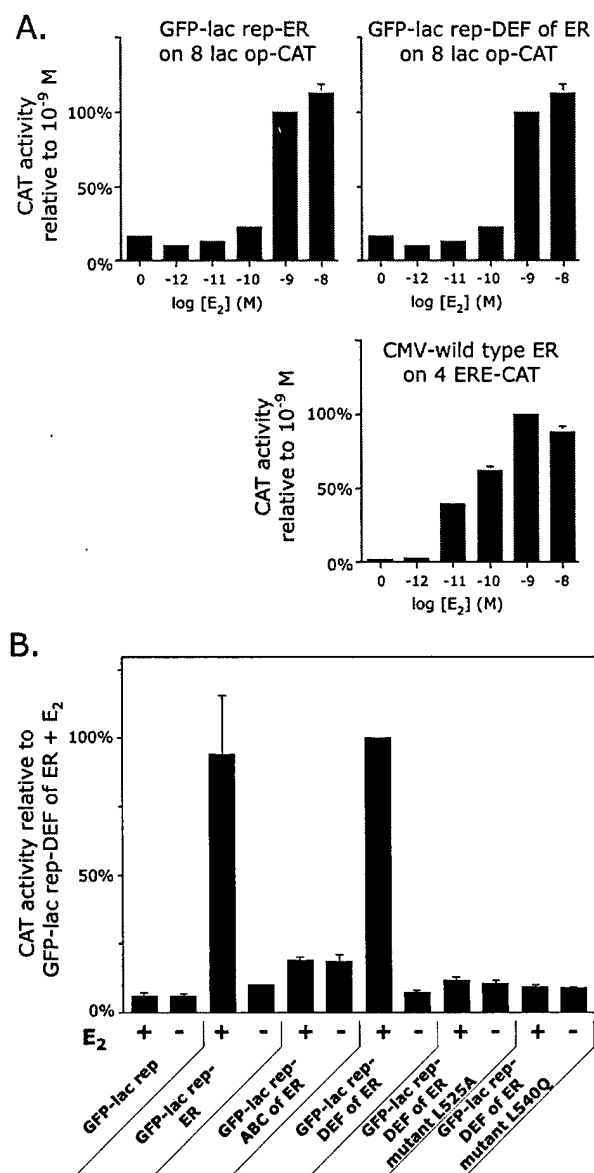


Figure 3

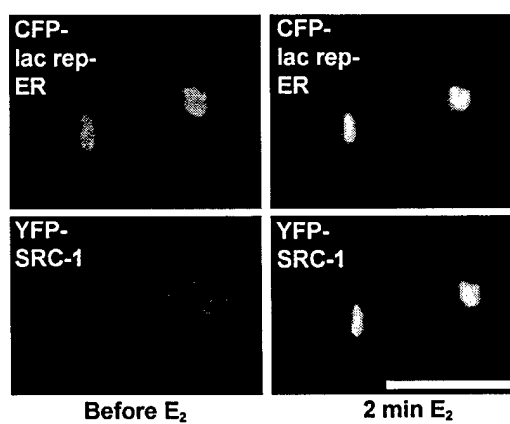


Figure 4

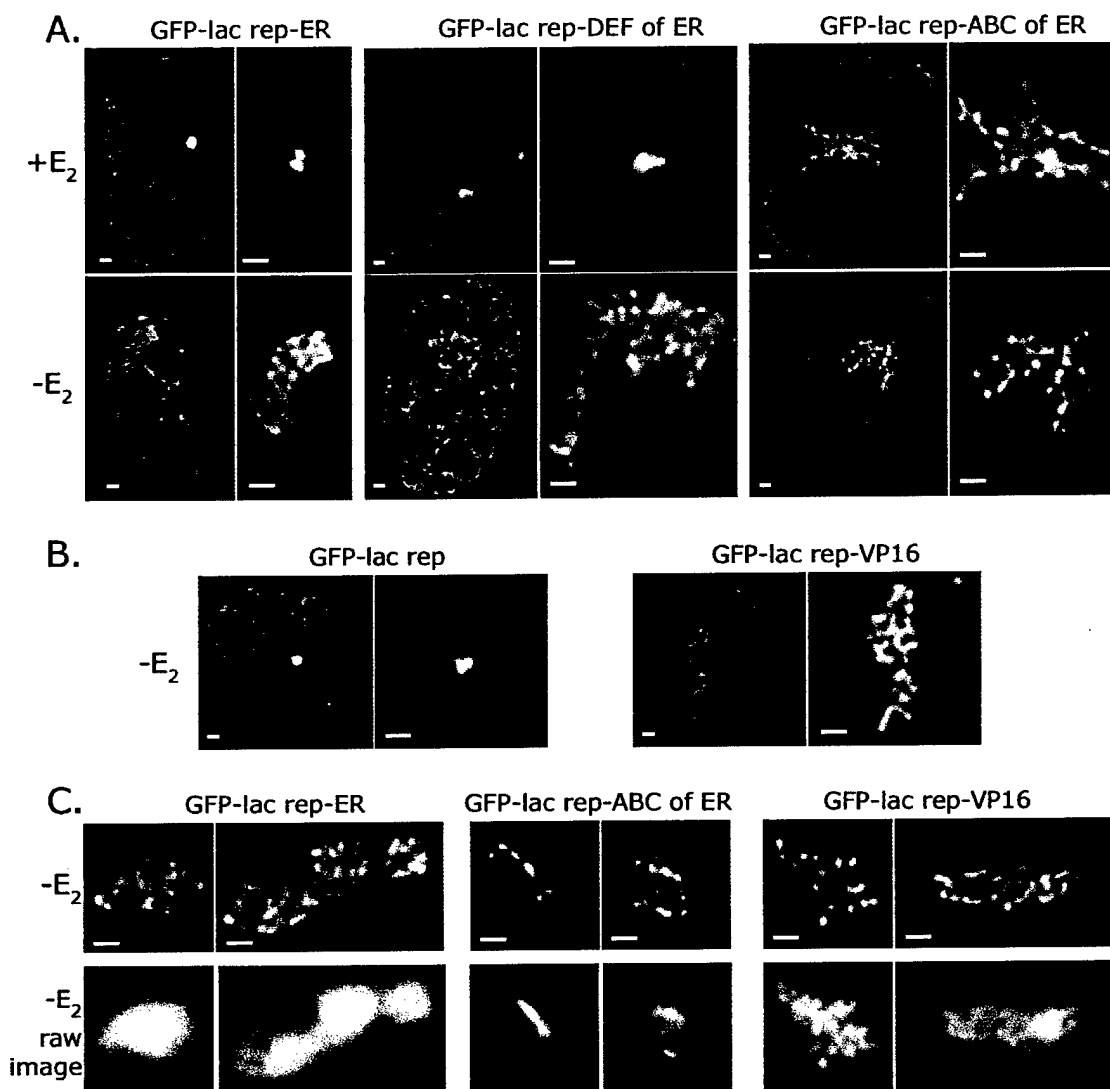


Figure 5

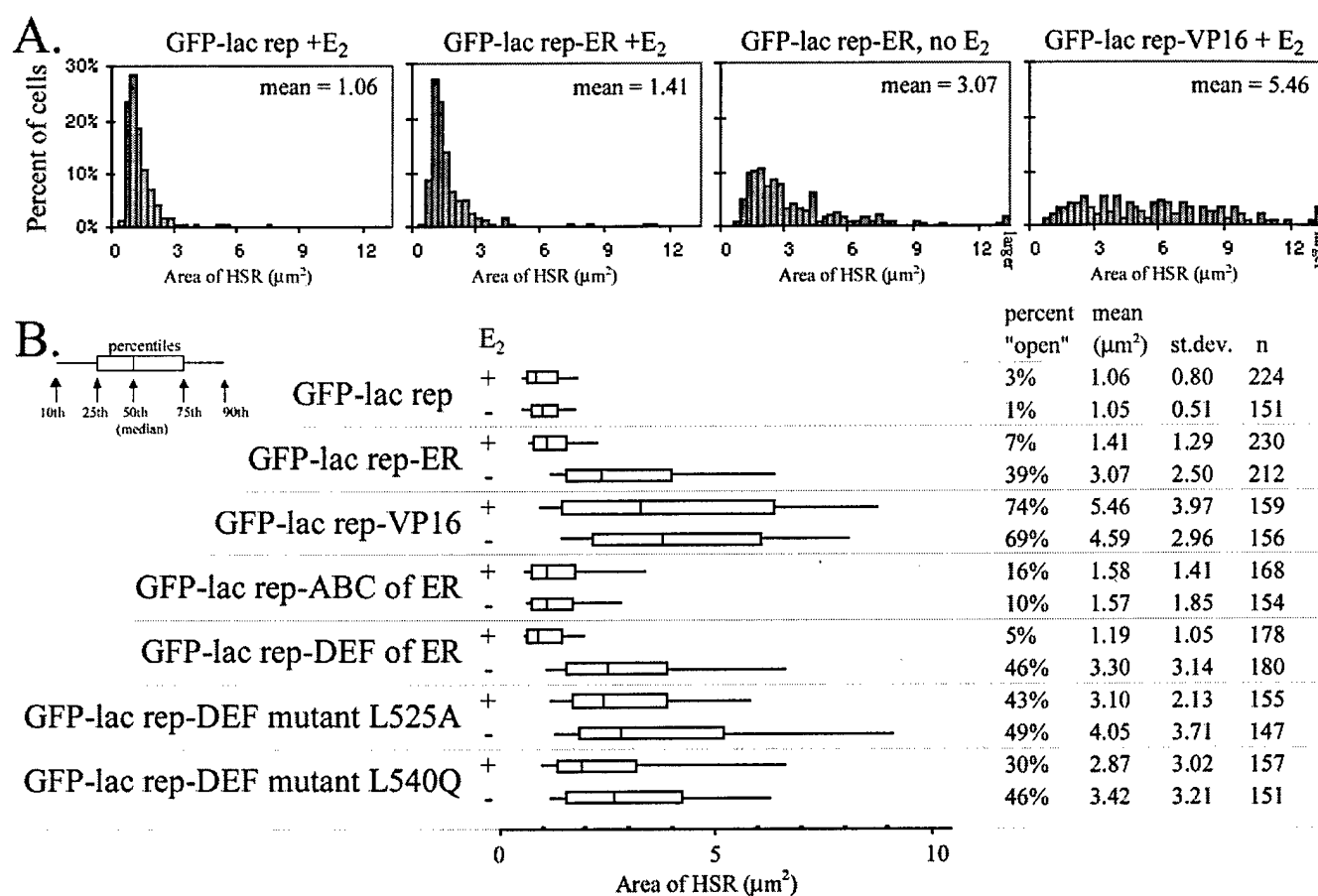


Figure 6

A03_1 cells: heterochromatic HSR

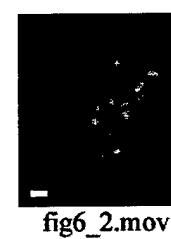
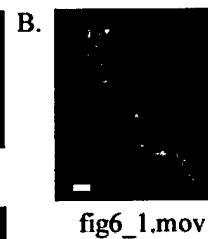
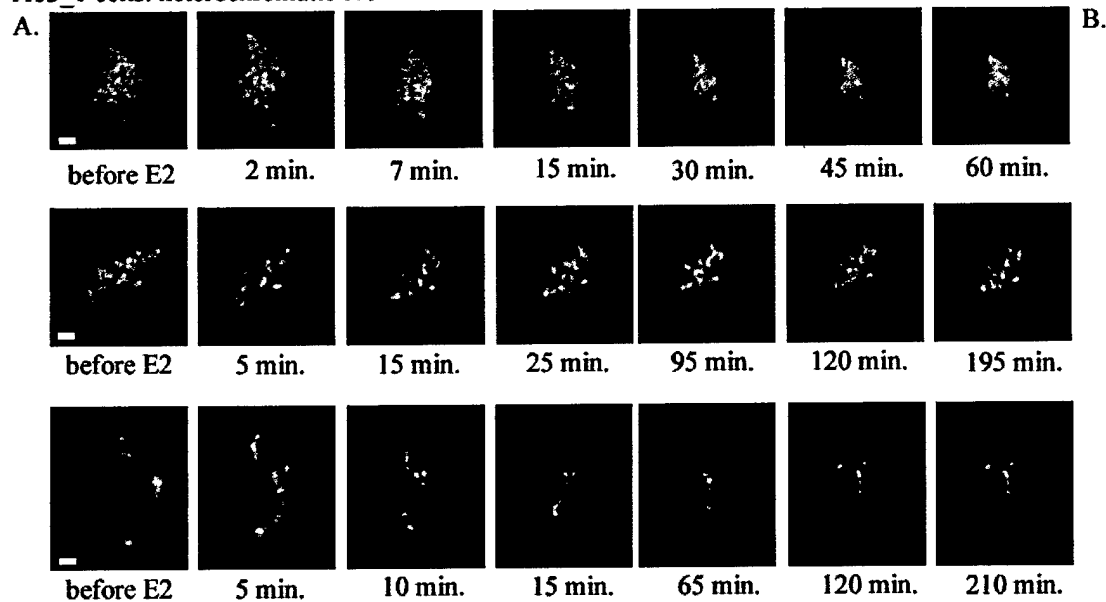


Figure 7

RRE_B1 cells: euchromatic HSR

